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(54) Title: MODIFIED INSULIN-LIKE GROWTH FACTORS

(57) Abstract

Modified forms of insulin-like growth factor (IGF) are provided which demonstrate improved pharmacological and biological properties. These modifications include IGF muteins produced by substituting or adding a cysteine in the amino acid sequence of native IGF as well as such muteins attached to polyethylene glycol (PEG) at the free cysteine site. The present invention further provides methods of making such modified forms. The IGF-PEG conjugates can be formulated into pharmaceutical compositions and used for the therapeutic treatment of IGF associated conditions.

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MODIFIED INSULIN-LIKE GROWTH FACTORS

Field of the Invention

This invention relates to the modification of polypeptides, and more particularly to the modification of insulin-like growth factors and to methods of making and using such modified polypeptides.

Background of the Invention

The insulin gene family, comprised of insulin, relaxin, insulin-like growth factors 1 and 2, and possibly the beta subunit of 7S nerve growth factor, represents a group of structurally related polypeptides whose biological functions have diverged as reported in Dull, et al., Nature 310:777-781 (1984).

Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are about 7-8 kilodalton proteins that are structurally related to each other and to insulin. IGF-1 and IGF-2 share about 70% amino acid identity with each other and about 30% amino acid identity with insulin. IGF-1 and IGF-2 are believed to have related tertiary structures as reported in PCT Application Publication No. WO 90/00569, published on January 25, 1990. The structural similarity between IGF-1 and IGF-2 permits both to bind to IGF receptors. Two IGF receptors are known to exist. IGF-1 and IGF-2 bind to the IGF type I receptor, while insulin binds with less affinity to this receptor. The type I receptor preferentially binds IGF-1 and is believed to transduce the mitogenic effects of IGF-1 and IGF-2. IGF-2 binds to the type I receptor with a 10-fold lower affinity than IGF-1. The second or type II IGF receptor preferentially binds IGF-2. Receptor binding is believed to be necessary for the biological activities of IGF-1 and IGF-2.

IGF-1 and IGF-2 are mitogenic for a large number of cell types, including fibroblasts, keratinocytes, endothelial cells and osteoblasts (bone-forming cells). IGF-1 and IGF-2 also stimulate differentiation of many cell types, e.g., synthesis and secretion of collagens by osteoblasts. IGF-1 and IGF-2 exert their mitogenic and cell differentiating effects by binding to the specific IGF cell surface receptors. IGF-1 also has been shown to inhibit protein catabolism in vivo, stimulate glucose uptake by cells and to promote survival of isolated neurons in culture. These properties have led to IGF-1 being tested as a therapeutic agent

for a variety of disease indications as reported in Froesch et al., Trends in Endocrinology and Metabolism, 254-260 (May/June 1990) and Cotterill, Clinical Endocrinology, 37:11-16 (1992).

In addition to specific cell surface receptors, there exist at least six distinct IGF binding proteins (IGFBP-1 through IGFBP-6) that circulate throughout the body. These proteins bind IGF-1 and IGF-2 with high affinity. The binding of IGF-1 and IGF-2 to binding proteins reduces the action of these IGFs on cells by preventing their interaction with cell surface IGF receptors. IGF binding proteins, particularly IGFBP-3, also function to prolong the circulating half-lives of IGF-1 and IGF-2 in the blood stream. In the absence of IGF binding proteins, the half-life of IGF-1 in blood is less than 10 minutes. In contrast, when IGF-1 is bound to IGFBP-3, its half-life in blood is lengthened to about 8 hours. The circulating half-life of IGF-1 bound to the other smaller binding proteins is about 30 minutes as reported in Davis et al., J. of Endocrinology, 123:469-475 (1989); Guler et al., Acta Endocrinologica, 121:753-758 (1989); and Hodgkinson et al., J. of Endocrinology, 123:461-468 (1989). When IGF is bound to binding proteins, it is unable to bind to the IGF receptors and is therefore, no longer active in the body. Decreased affinity to binding proteins allows more of the IGF to be active in the body. Situations where this decreased affinity to binding proteins may be useful include, for example, cachexia, osteoporosis, and peripheral neuropathies.

Furthermore, the therapeutic utility of IGF can be modified by the presence or absence of these IGF binding proteins, which may potentiate or inhibit the beneficial effects of IGF. The levels of certain IGF binding proteins can vary greatly, depending upon the disease state. For example, IGFBP-1 levels are very high in diabetes patients, whereas they are nearly undetectable in normal patients as reported in Brismar et al., J. of Endocrinological Investigation, 11:599-602 (1988); Suikkari et al., J. of Clinical Endocrinology and Metabolism, 66:266-273 (1988); and Unterman et al., Biochem. Biophys. Res. Comm., 163:882-887 (1989). IGFBP-3 levels are reduced in severely ill patients such as those that have undergone major surgery as reported in Davies et al., J. Endocrinology, 130:469-473 (1991); Davenport et al., J. Clin. Endocrin. Metab., 75:590-595

(1992). The reduced levels of IGFBP-3, and consequent shorter circulating half-life of IGF-1, may contribute to the cachexia (weight loss) seen in these patients.

Insulin-like growth factor 1 (IGF-1), also known as somatomedin C, has long been studied for its role in the growth of various tissues. Its role as a useful therapeutic agent for several disease conditions has been suggested. Significantly reduced levels of IGF-1 were found in 23 patients with varying extent and severity of burns as reported in Moller et al., Burns, 17(4):279-281 (1991). A marked rise in serum type III procollagen, a marker of bone formation, occurred after one week of administration of recombinantly produced IGF-1 to patients with dwarfism otherwise non-responsive to growth hormone as reported in Laron et al., Clinical Endocrinology, 35: 145-150 (1991). The effects of the infusion of IGF-1 in a child with Laron Dwarfism is described in Walker et al., The New England Journal of Medicine, 324(21):1483-1488 (1991). Increased weight gain, nitrogen retention and muscle protein synthesis following treatment of diabetic rats with IGF-1 or IGF-1 having a deletion of the first three amino acids ordinarily found in IGF-1 (referred to as "(des1-3)IGF-1") were demonstrated in Tomas et al., Biochem. J., 276:547-554 (1991). Growth restoration in insulin-deficient diabetic rats by administration of recombinantly produced human IGF-1 is reported in Scheiwiller et al., Nature, 323:169 (1986). IGF-1 and (des1-3)IGF-1 enhance growth in rats after gut resection, as reported in Lemmey et al., Am. J. Physiol., 260 (Endocrinol. Metab. 23) E213-E219 (1991). A combination of platelet-derived growth factor and insulin-like growth factors, including IGF-1, enhanced periodontal regeneration in beagle dogs as reported by Lynch et al., J. Clin. Periodontal, 16:545-548 (1989). The synergistic effects of platelet-derived growth factor and IGF-1 in wound healing are reported in Lynch et al., Proc. Natl. Acad. Sci., 84:7696-7700 (1987). The effects of IGF-1 and growth hormone on longitudinal bone growth in vitro are set forth in Scheven and Hamilton, Acta Endocrinologica (Copenhagen) 124:602-607 (1991). In vivo actions of IGF-1 on bone formation and resorption in rats are shown in Spencer et al., Bone, 12:21-26 (1991). The use of IGF-1 and IGF-2 for enhancing the survival of non-mitotic, cholinergic neuronal cells in a mammal is described in U.S. Patent 5,093,317 to Lewis et al. In addition, PCT Application Publication No. WO 92/11865 published

on July 23, 1992, describes the use of IGF-1 for the treatment of cardiac disorders.

Various modifications to the naturally occurring or wild-type IGF-1 have been described. For example, the naturally occurring variant of IGF-1 missing from the first three N-terminal amino acids, (des1-3)IGF-1, was discovered in cerebral spinal fluid and in colostrum as reported in Sara, et al., Proc.Natl.Acad.Sci., 83:4904-4907 (1986) and Francis et al., Biochemical Journal, 251:95-103 (1988). In vitro studies have shown that this variant is equipotent to IGF-1 in binding to IGF cell surface receptors and in stimulating cell mitogenesis. Thus, the first three amino acids of IGF-1 appear to be nonessential for the binding of IGF-1 to its specific cell surface receptors. (Des1-3)IGF-1 was found to have greatly reduced affinity (100-fold less) for certain IGF binding proteins, specifically IGFBP-1 and IGFBP-2, as reported in Forbes et al., Biochem. Biophys. Res. Comm., 157:196-202 (1988); and Carlsson-Skwirut et al., Biochim. Biophys. Acta, 1101:192-197 (1989). The binding of IGFBP-3 to (des1-3)IGF-1 also is affected, being reduced by two to three fold. Animal studies have shown that (des1-3)IGF-1, when given by continual subcutaneous infusion, is more potent than IGF-1 in stimulating a number of anabolic functions, such as growth, reported in Cascieri et al., J. Endocrinology, 123:373-381 (1988) and Gillespie et al., J. Endocrinology, 127:401-405 (1990). The enhanced properties of (des1-3)IGF-1 are believed to result from its reduced affinity for IGF binding proteins, reported in Carlsson-Skwirut et al., Biochim. Biophys. Acta, 1101:192-197 (1989). The reduced affinity of (des1-3)IGF-1 for IGF binding proteins results in (des1-3)IGF-1 having a shorter circulating half-life than wild type IGF-1 as reported in Cascieri et al., J. Endocrinology, 123:373-381 (1988). Therefore, (des1-3)IGF-1 must be administered by continual infusion or by multiple daily injections in order to effect its enhanced potency.

PCT Application Publication No. WO 89/05822 published on June 29, 1989, describes other modifications of IGF-1. This application describes substituting the third amino acid from the N-terminal end of the naturally occurring IGF-1 with glycine, glutamine, leucine, arginine or lysine to form IGF-1

muteins. This reference however does not teach replacing the third amino acid with cysteine.

The potential therapeutic usefulness of IGF-1 and (des1-3) IGF-1 is limited by their short circulating half-lives to situations when the proteins can be administered by continual infusion or by multiple daily injections to achieve their maximum therapeutic potential. As an example, Woodall et al., Horm. Metab. Res., 23: 581-584 (1991), reports that the same total dose of IGF-1 administered four times daily by subcutaneous injection was far superior in stimulating growth in mutant lit/lit mice (growth hormone deficient mice) than was the same total dose administered once daily.

There are many cases in which it would be preferable to administer IGF-1 in a single once-a-day injection or in a single injection given once every several days. For injectable drugs, patient compliance is expected to be higher for drugs that can be administered once a day rather than several times a day. In order for IGF-1 to be therapeutically effective when given once a day or once every few days, methods must be developed to increase its circulating half-life.

Increasing the molecular weight of a protein by covalently bonding an inert polymer chain such as polyethylene glycol (PEG) to the protein is known to increase the circulating half-life of the protein in the body. See, for example, Davis et al, Biomedical Polymers: Polymeric Materials and Pharmaceuticals for Biomedical Use, p. 441-451 (1980). However, since multiple PEG molecules can bind to each protein molecule, and because there are typically a large number of sites on each protein molecule suitable for binding to several PEG molecules using known methods, there has been little success in attaching PEG to yield homogeneous reaction products. See Goodson et al, Biotechnology, 8:343 (1990), and U.S. Patent 4,904,584. This lack of site attachment specificity can give rise to a number of problems, including loss of activity of the protein.

Thus, a need exists for prolonging the circulating half life of IGF without compromising its usefulness as a therapeutic agent. The present invention satisfies this need by increasing the molecular weight of the IGF. This is accomplished by providing site directed attachment of PEG to IGF.

Summary of the Invention

The invention relates to various modified forms of IGF. One type of modified IGF, referred to as muteins, is produced by replacing cysteine residues for specific amino acids in the wild type molecule, or by inserting cysteine residues between amino acids in the wild type molecule. Cysteine residues which are not involved in intramolecular disulfide bonds are considered to be "free". The free cysteine residues can be substituted or inserted in regions of the IGF molecule that are exposed on the protein's surface. The free cysteine serves as the attachment site for the polyethylene glycol (PEG) molecules to IGF, resulting in pegylated molecules. Attachment of the PEG molecule to a mutein creates a further modified form of IGF, or IGF-PEG conjugate of defined structure, where the PEG is attached to the IGF at a predetermined site.

Thus, the present invention is directed to a polyethylene glycol (PEG) conjugate comprising PEG and a mutein of IGF, and particularly IGF-1, where the PEG is attached to the mutein at a free cysteine in the N-terminal region of the mutein. PEG can be attached to the free cysteine through an activating group selected from maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane and 5-pyridyl. The cysteine can be inserted or substituted within the first twenty amino acids of wild-type IGF-1. A suitable PEG can have a molecular weight of 5 kDa, 8.5 kDa, 10 kDa or 20 kDa. The PEG conjugate of the present invention can also contain a second protein to form a dumbbell. Methods of making the PEG conjugates are also provided.

It has been discovered that this IGF-PEG conjugate, when compared to wild type IGF, exhibits decreased affinity to binding proteins without significantly reduced biological activity. Thus, IGF can be administered to patients less frequently with equal or better effectiveness than in the past.

The present invention is further directed to muteins of IGF having a free cysteine in the N-terminal region of the mutein. The muteins can be produced by recombinant methods.

Also provided in the present invention are pharmaceutical compositions comprising the IGF-PEG conjugate and methods of using the IGF-PEG conjugate to treat a patient having or potentially having an IGF associated condition.

Detailed Description of the Invention

The present invention is directed to modified forms of insulin-like growth factors (IGF) that provide beneficial properties not exhibited by wild-type IGF. The modified forms of IGF include muteins of these growth factors, containing at least one free cysteine. Conjugates containing the IGF muteins attached to polyethylene glycol (PEG) are also considered modified forms of IGF.

Terms used throughout this specification are defined as follows:

The term "IGF" refers to any polypeptide that binds to the IGF type I Receptor, including, for example, IGF-1, IGF-2, (des1-3)IGF-1, and insulin. This hormone family is described in Blundell and Humbel, Nature, 287:781-787 (1980). Due to this common receptor binding, the teachings of the present invention which are described with respect to IGF-1 are intended to encompass IGF-2, des(1-3) IGF-1, and insulin also.

The term "wild type IGF-1" refers to the unmodified or naturally-occurring 70 amino acid form of IGF-1. This term is used interchangeably with "IGF-1" and "naturally occurring IGF-1".

The term "IGF-PEG conjugate" refers to an IGF molecule attached to a polyethylene glycol molecule. This is also referred to as a "Peg conjugate".

The term "N-terminal region" refers to approximately the first twenty amino acids from the N-terminus of IGF or an IGF mutein, and up to twelve amino acids preceding the first amino acid of the N-terminus of IGF.

The term "N-terminus" refers to the first amino acid at the N-terminal region in the sequence of wild-type IGF, for example, glycine in IGF-1.

The term "mutein" refers to a modified form of IGF, which has been modified to contain a free cysteine in the N-terminal region.

The term "activating group" refers to a site on the PEG molecule which attaches to the mutein.

The term "pharmaceutically acceptable carrier" refers to a physiologically-compatible, aqueous or non-aqueous solvent.

The term "free cysteine" refers to any cysteine residue not involved in an intramolecular disulfide bond.

The term "IGF associated condition" refers to an existing or potential adverse physiological condition which results from an over-production or underproduction of IGF, IGF binding protein or IGF receptor, inappropriate or inadequate binding of IGF to binding proteins or receptors and any disease in which IGF administration alleviates disease symptoms. An IGF associated condition also refers to a condition in which administration of IGF to a normal patient has a desired effect.

The term "patient" refers to any human or animal in need of treatment for an IGF associated condition.

The IGF muteins of the present invention are produced by modifying wild-type IGF, particularly at the N- terminal region of the protein. Such modifications can be substitutions or additions of at least one cysteine residue in the N-terminal region of IGF. An IGF mutein can be produced by replacing a specific amino acid with a cysteine in approximately the first twenty amino acids of the N-terminus of wild-type IGF, such as, for example, substituting one of the first three amino acids of IGF-1 with a cysteine residue. The amino acid sequence of IGF-1 starting from the N-terminal end is: G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A (SEQ.ID.NO. 1).

Alternatively, the IGF muteins of the present invention can be produced by adding at least one cysteine residue in front of the first amino acid of IGF. For example, a cysteine residue can be inserted in front of and adjacent to the first amino acid of IGF. For muteins produced by E. coli, the free cysteine can appear between Met and the first amino acid of IGF. A free cysteine residue can also appear in a group of about twelve or less amino acids inserted before the first amino acid of IGF to form a longer IGF mutein.

The free cysteine residues are located in regions of the IGF-1 molecule exposed to the protein's surface. The N-terminal region is involved in the binding of the IGF to binding proteins, but is not involved in binding of IGF to cell surface IGF receptors.

A mutein is referred to as "a cysteine mutein of IGF-1." The free cysteine residue acts as the attachment site for covalent linkage of the activating group

on the polyethylene glycol. The newly created molecule comprising the cysteine mutein of IGF with the PEG attached is referred to as a "PEG conjugate of IGF".

The IGF muteins of the present invention can be prepared by methods well known to one skilled in the art. Such methods include mutagenic techniques for replacement or insertion of an amino acid residue, as described, for example, in U.S. Patent 4,518,584, incorporated herein by reference. The muteins produced by mutagenic techniques can then be expressed as recombinant products according to procedures known to those skilled in the art. The muteins can alternatively be synthesized by conventional methods known in the art. The IGF muteins can also be prepared according to the methods and techniques described in the examples set forth below.

The present invention also provides IGF-PEG conjugates and methods of making such conjugates by attaching the IGF muteins to polyethylene glycol to increase the circulating half-life of the molecule in the body as well as decrease its affinity to IGF binding proteins.

In the present invention long chain polymer units of polyethylene glycol (PEG) are bonded to the mutein via a covalent attachment to the sulfhydryl group of a free cysteine residue on the IGF mutein. Various PEG polymers with different molecular weights, 5.0 kDa (PEG_{5000}), 8.5 kDa (PEG_{8500}), 10 kDa ($PEG_{10,000}$), and 20 kDa ($PEG_{20,000}$) can be used to make the IGF-PEG conjugates. In order to obtain selectivity of reaction and homogenous reaction mixtures, it is useful to use functionalized polymer units that will react specifically with sulfhydryl groups. The functional or reactive group attached to the long chain polyethylene glycol polymer is the activating group to which the IGF mutein attaches at the free cysteine site. Useful activating groups include, for example, maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane, or 5-pyridyl.

In another embodiment, polyethylene glycol (PEG) polymers containing two activating groups can be used to create "dumbbell" type molecules containing two IGF muteins attached to one molecule of PEG at each end of the PEG molecule. For example, PEG bis-malemide (a polyethylene glycol polymer containing a maleimide activating group on each end of the PEG molecule) can be used to create these "dumbbell" type molecules. These dumbbell molecules can

also comprise a single IGF mutein covalently attached via PEG to a second protein or peptide of different structure. The second protein or peptide can be, for example, a growth factor such as platelet-derived growth factor, or fibroblast growth factor.

One skilled in the art can readily determine the appropriate pH, concentration of protein, and ratio of protein:PEG necessary to produce a useful yield of either mono-pegylated IGF-1 (IGF-PEG), or dumbbell IGF-1 (IGF-PEG-IGF, IGF-PEG-PDGF, or IGF-PEG-FGF) using conventional methods known to one skilled in the art for making these determinations.

The present invention further provides a pharmaceutical composition containing the IGF-PEG conjugates in a pharmaceutically acceptable carrier. One carrier is physiological saline solution. Other pharmaceutically acceptable carriers can also be used. In one embodiment, it is envisioned that the carrier and the IGF-1 constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier may be either aqueous or non-aqueous in nature. In addition, the carrier may contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the IGF-1. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dose or multi-dose form.

Once the pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations may be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The storage of such formulations can be at temperatures at least as low as 4°C and preferably at -70°C. Formulations containing IGF-1 can also be stored and administered at or near physiological pH. It is presently believed that storage and administration in a formulation at a high pH (i.e. greater than 8) or at a low pH (i.e. less than 5) is undesirable.

The pharmaceutical composition of the present invention can be used to treat a patient having or potentially having an IGF associated condition. Some of these conditions may include, for example, dwarfism, diabetes, periodontal disease and osteoporosis. The pharmaceutical composition of the present invention can also be used to treat a condition in which administration of IGF to a normal patient has a desired effect; for example, using IGF-1 to enhance growth of a patient of normal stature.

The manner of administering the formulations containing the IGF-PEG conjugate can be via an intraarticular, subcutaneous, intramuscular or intravenous injection, suppositories, enema, inhaled aerosol, or oral or topical routes. To achieve and maintain the desired effective dose of IGF-1 mutein, repeated subcutaneous or intramuscular injections may be administered. Both of these methods are intended to create a preselected concentration range of IGF-1 mutein in the patient's blood stream. It is believed that the maintenance of circulating concentrations of IGF-1 mutein of less than 0.01 ng per ml of plasma may not be effective, while the prolonged maintenance of circulating levels in excess of 100 ug per ml may be undesirable. The frequency of dosing will depend on pharmacokinetic parameters of the IGF-PEG conjugate in the formulation used.

The following examples are intended to illustrate the present invention and are not intended to be limiting.

EXAMPLE 1

A. Construction of the IGF-1 gene

The IGF-1 gene was assembled in two stages. Initially, the DNA sequence encoding IGF-1 was joined to DNA sequences encoding the secretory leader sequence of the E. coli OMP A protein (ompAL). This gene fusion was constructed in order to determine whether IGF-1 could be efficiently secreted from E. coli. A second construct, in which IGF-1 is expressed as an intracellular protein in E. coli, was created by deleting DNA sequences encoding the OmpA leader sequence and replacing them with DNA sequences that allow intracellular expression of IGF-1.

B. Construction of the OmpAL-IGF-1 gene fusion

The four synthetic oligonucleotides labeled OmpA1U (SEQ ID NO:2), OmpA2U (SEQ ID NO:3), OmpA1L (SEQ ID NO:4) and OmpA2L (SEQ ID NO:5), were annealed pairwise (1U + 1L and 2U + 2L) and the pairs ligated together. All four of these oligonucleotides were synthesized using DNA synthesizers purchased from Applied Biosystems (Models 391 and 380A). The ligation mixture was then digested with the restriction enzyme HaeIII. The resulting BamHI/HaeIII restriction fragment coding for a translational start signal and the first 21 amino acids of the ompA signal sequence was purified. This DNA fragment was mixed with BamHI + PstI-digested PUC18 DNA (commercially available from Boehringer Mannhein Biochemicals, Indianapolis, IN) and the two synthetic oligonucleotides [IGF-1 (1-14) U + L] (SEQ ID NO:6 and SEQ ID NO:7) were ligated together. The ligation mixture was used to transform E. coli strain JM109 (commercially available from New England Biolabs, Beverly, MA) and individual colonies isolated. These plasmids (OmpALIGF-1pUC18) have a translational start signal followed by DNA sequences encoding the OmpA signal sequence and the first 14 amino acids of IGF-1.

An M13 phage containing DNA sequences encoding amino acids 15 through 70 of IGF-1 was created by ligating together the two complementary pairs of oligonucleotides (IGF1U + 1L and IGF2U + 2L) (SEQ ID NO:8 and SEQ ID NO:9) and cloning the DNA fragment into PstI + HindIII-digested M13 mp19 DNA (commercially available from New England Biolabs, Beverly, MA). Double-stranded DNA was purified from a phage clone and the PstI/HindIII fragment encoding amino acids 15-70 of the IGF-1 protein were isolated. This DNA fragment was ligated together with PstI + HindIII-digested plasmid OmpALIGF-1pUC18 DNA and used to transform E. coli strain JM107 (commercially available from GIBCO BRL, Gaithersburg, MD). The BamHI/HindIII fragment containing the IGF-1 gene fused to the OmpAL sequence was isolated and cloned into the BamHI + HindIII generated site of plasmid pT3XI-2 (described in PCT Application publication WO 91/08285 published on June 13, 1991). The completed plasmid containing the ompAL-IGF-1 gene fusion is called pT3XI-2 ø10C(TC3)ompALIGF-1.

C. Construction of the Methionyl-IGF-1 gene

The BamHI/HindIII fragment containing the OmpAL-IGF-1 gene fusion described above was purified from plasmid pT3XI-2ø10C(TC3)ompALIGF-1 and digested with HinfI. The approximate 200 bp HinfI/HindIII DNA fragment was mixed with the annealed, complementary synthetic oligonucleotides (MetIGF1U + 1L) (SEQ ID NO:10 and SEQ ID NO:11) and ligated with BamHI + HindIII-digested plasmid pT3XI2 DNA, and used to transform E. coli JM107. The completed plasmid construct is called ø10C(TC3)IGF-1pT3XI-2 and contains an extra alanine residue in between the initiator methionine and the beginning of the IGF-1 sequence. The BamHI/HindIII fragment containing the mutant IGF-1 gene was isolated and ligated into the BamHI + HindIII generated site of plasmid pT5T (described in Nature, Vol. 343, No. 6256, pp. 341-346, 1990). The ligation mixture was used to transform E. coli BL21/DE3 described in US Patent 4,952,496 and the resulting individual colonies were isolated. This construct was named ø10C(TC3)IGF-1pT5T.

The extra alanine codon was removed by in vitro mutagenesis. In vitro mutagenesis was performed using a Muta-Gene kit purchased from Bio-Rad Laboratories (Richmond, CA). The mutagenesis procedure followed was essentially that described in the instructions that accompany the kit. Plasmid ø10C(TC3)IGF-1pT3XI-2 was digested with BamHI + HindIII and the _200 bp DNA fragment containing the mutant IGF-1 gene was purified and cloned into the BamHI and HindIII sites of plasmid M13 mp19.

Uracil-containing single-stranded template DNA was prepared following propagation of the phage in E. coli strain CJ236 (supplied with Muta-Gene Kit purchased from Bio-Rad Laboratories, Richmond, CA). The oligonucleotide used for mutagenesis had the sequence: 5' - GATGATTAAATGGGTCCGGAGACT - 3' (SEQ ID NO 12). The mutagenesis reaction product was used to transform E. coli strain JM109 and individual plaques picked.

Double-stranded replicative form DNA from individual phages was isolated, digested with BamHI + HindIII and the _200 bp fragment containing the IGF-1 gene purified. The purified DNA was cloned into the BamHI + HindIII generated site of plasmid pT5T and used to transform E. coli strain BL21/DE3. One

bacterial colony with the correct plasmid was named ø10(TC3)mutIGF-1pT5T. Several isolates were sequenced, and all were correct.

EXAMPLE 2

Construction of IGF-1 Muteins

Four muteins of IGF-1 were constructed. Three of the muteins replaced each of the first three amino acids of IGF-1 with a cysteine residue. These muteins are referred to as C1, C2, and C3, respectively. The fourth mutein introduced a cysteine residue between the N-terminal methionine residue and the first amino acid of IGF-1. This mutein is referred to as -1C.

The muteins of IGF-1 were made using the polymerase chain reaction (PCR) technique as described below. The starting plasmid used for the mutagenesis experiments was ø10(TC3)mutIGF-1pT5T, which is described in Example 1. This plasmid contains DNA sequences encoding an initiator methionine followed by the sequence of the natural human IGF-1 protein. Mutant IGF-1 DNA sequences were amplified from this gene using a 5' mutagenesis oligonucleotide that contained the desired mutation and a 3' oligonucleotide of correct sequence. The 5' mutagenesis oligonucleotides were designed so that they incorporated the first methionine of the gene as part of an Nde I restriction enzyme cleavage site (CATATG). Each mutagenesis oligonucleotide contained the desired mutation followed by 15 to 21 nucleotides that were a perfect match to the IGF-1 gene sequences in plasmid ø10(TC3)mutIGF-1pT5T. The 3' oligonucleotide was 25 nucleotides long and was designed to encode the last 6 codons of IGF-1 and to contain the Hind III site that follows the stop codon.

TABLE 1

mutein	a.a. sequence	oligo	sequence
wild type	M G P E T L C...	IGF(85p)28	5'GGGCATATGGGTCCGGAGACTCTGTGCG3'
-1C	M C G P E T L C...	IGF(-1Cp)33	5'GGGCATATGTGTGGTCCGGAGACTCTGTGCGGC3'
C1	M C P E T L C...	IGF(C1p)33	5'GGGCATATGTGCCCGGAGACTCTGTGCGGCGCA3'
C2	M G C E T L C...	IGF(C2p)31	5'GGGCATATGGGTTGTGAGACTCTGTGCGGCG3'
C3	M G P C T L C...	IGF(C3p)33	5'GGGCATATGGGTCCGTGCACTCTGTGCGGCGCA3'
3' oligo (for all muteins)		IGF(262p)25	5'CCCAAGCTTAAGCGCTTTTAGCCGG3'

(overlaps with IGF sequence are underlined)

(SEQ ID NOS. 13 through 18 respectively)

Polymerase chain reaction (PCR) was performed in 100ul reactions containing 20 mM Tris pH 8.8, 10 mM KCl, 6 mM (NH4)2SO4, 1.5 mM MgCl2, 0.1% Triton X-100 using 20 pmole of each oligo and approximately 1ng of plasmid ø10(TC3)mutIGF-1pT5T as template DNA. 0.5ul (1.25 units) of Pfu polymerase (Stratagene, San Diego, CA) was added after the first denaturation step with the tubes held at 65°C. The reactions were overlayed with 2 drops of mineral oil at that time. The reactions were cycled 30 times for 1 min. at 95°C, 1 min. at 65°C, and 1 min. at 72°C in an Ericomp Twinblock™ thermal cycler (Ericomp, San Diego, CA). After the last cycle the reactions were held at 72oC for 10 minutes.

After PCR, 80ul of the reactions were phenol extracted one time then ethanol precipitated. The precipitated DNA was resuspended in 80ul of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 20ul was digested with Nde I and Hind III and electrophoresed on a 1.5% agarose gel. The amplified DNA bands that ran at approximately 120bp were eluted using NA45 paper (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. The eluted DNA was resuspended in 20ul of TE buffer and 2ul was ligated to gel-purified Nde I and Hind III digested plasmid pT5T in a volume of 20ul. Plasmid pT5T is described in Example 1. The ligation reactions were used to transform E. coli strain BL21/DE3 and colonies selected on LB agar plates containing 50ug/ml of ampicillin. Mini plasmid DNA preps were made from several colonies from the transformation plates. The DNAs were digested with Eco RV and Hind III to determine which transformants contained IGF-1 DNA inserts. Plasmids containing IGF-1 DNA inserts were sequenced to verify that the inserts were correctly mutated (the entire IGF gene was sequenced for each mutein).

Preliminary growth studies were performed by growing a representative transformant for each mutein in Luria Broth + 12ug/ml tetracycline to an approximate OD_{600} of about 1.0. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to 1 mM to induce expression of T7 polymerase and the subsequent transcription and translation of the IGF muteins. Approximately 0.1 OD unit of cells were lysed in SDS sample buffer by boiling for two minutes and electrophoresed on a 16% polyacrylamide SDS gel. The gel was stained with

Coomassie blue. IGF-1 protein bands of the expected size which is approximately 7-8 kDa. were observed in the lanes loaded with induced cells for each mutein as well as for the wild-type control.

EXAMPLE 3

Expression, Refolding, and Purification of Muteins

Although the following is written for the C3 mutein, the same procedure applies to other muteins contemplated by the instant invention. The only difference is in the starting cells used.

E. coli cells expressing the IGF-1 C3 mutein were suspended in Buffer A (50 mM Tris, pH 7.5, 20 mM NaCl and 1 mM dithiothreitol (DTT) at a concentration of 40 ml Buffer A to 10 g cell paste, and disrupted at 1800 psi using a French pressure cell (SLM Instruments, Inc., Urbana IL). The suspension was centrifuged at 20,000 X g for 30 min, and aliquots of the pellet and supernatant analyzed by SDS-PAGE. A major band corresponding to the IGF-1 C3 mutein was present in the pellet, but not the supernatant. The pellet was suspended in Buffer A at a concentration of 40 ml Buffer A to 10 g cell paste, and re-centrifuged at 20,000 X g for 30 min. This wash procedure was repeated 2 times. The final pellet containing the IGF-1 C3 mutein was suspended in 6 M guanidine, 50 mM Tris, pH 7.5, 6 mM DTT at a concentration of 25 ml to 10 g cells using a ground glass homogenizer. The suspension was incubated at room temperature for 15 min. The undissolved protein was removed by centrifugation at 20,000 X g for 30 min. The final concentration of the C3 mutein was 1.0 mg/ml. SDS-PAGE analysis of the pellet and supernatant following the procedure of Example 2 showed that IGF-1 C3 mutein was present in the supernatant only.

The denatured and reduced IGF-1 mutein was subjected to the following three-step refolding procedure:

1) Oxidized glutathione, the mixed-disulfide producing reagent (GSSG), was added to the supernatant to a final concentration of 25 mM, and incubated at room temperature for 15 min.

2) The solution was then diluted 10 fold gradually with 50 mM Tris, pH 9.7, and phenylmethylsulfonylfluoride and cysteine were added to

final concentrations of 1mM and 5 mM, respectively. Final concentration of protein was 100ug/ml.

3) The refolding mixture was incubated overnight at 4oC, and then centrifuged at 20,000 x g for 15 min. SDS-PAGE analysis of the pellet and supernatant showed that the supernatant was composed of relatively homogeneous IGF-1 C3 mutein.

Aliquots (50ul) of the supernatant were diluted to 200ul with Buffer B (0.05% TFA), injected onto a reverse phase column (RP-4, 1 x 250mm, Synchrom, Lafayette, IN), and eluted with 80% acetonitrile, 0.042% TFA (Buffer C) using a linear gradient (increase of 2% Buffer C/min) at a flow rate of 0.25 ml/min.

A single major peak representing refolded IGF-1 C3 mutein eluted at 26.5 min. Refolded IGF-1 C2 mutein eluted at 26.0 min. The retention times of refolded IGF-1 C3 and IGF-1 C2 muteins shifted to 32.2 min and 31.7 min, respectively, after being completely reduced and denatured in 5 M guanidine, 50 mM Tris pH 7.5, 100 mM DTT. These results indicate that both the C3 and C2 muteins refold to a single major species under the conditions described. N-terminal sequence analysis of IGF-1 C3 mutein eluting at 26.6 min gave the sequence MGPCTLC... (SEQ ID NO. 19) confirming that a cysteine residue has been substituted for glutamic acid in the 3 position of the N-terminal sequence of natural human IGF-1. An extra methionine residue is present at the N-terminus of the recombinant protein expressed by E coli. N-terminal sequence analysis of IGF-1 C2 mutein eluting at 26.0 min gave the sequence GCETLC... (SEQ ID NO. 20) confirming that a cysteine residue has been substituted for proline in the 2 position of the N-terminal sequence of natural human IGF-1.

EXAMPLE 4

Isolation of Refolded IGF-1 Mutein

Refold mixtures (435 ml) prepared from 20g of E. coli paste containing either the refolded C3 or C2 mutein of Example 3 were concentrated to 100ml, acidified to pH 5.5 with 5M HCl, dialyzed against 20 mM sodium acetate, pH 5.5, and loaded onto an S-Sepharose (Pharmacia/LKB, Piscataway, NJ) column (1.6

X 15 cm) previously equilibrated with the sodium acetate buffer described above. The bound protein was eluted with a 300 ml linear gradient from 0 to 0.5M NaCl. Three ml fractions were collected. A single major protein peak eluted at 0.2-0.3M NaCl. Aliquots (100 ul and 25 ul) of each peak were analyzed separately by reverse phase (RP-4 1 X 250 mm) and gel filtration chromatography (Superdex 75 3.2 X 300 mm, Pharmacia/LKB, Piscataway, NJ). Fractions containing predominantly correctly refolded (determined from RP-4 analysis), and monomeric (determined from gel filtration analysis) C3 mutein were pooled, concentrated to 2.0 mg protein/ml, and 2.0-5.0 ml aliquots were loaded onto a Sephacryl S-100 (Pharmacia/LKB, Piscataway, NJ) gel filtration column (2.6 X 100 cm) previously equilibrated with 20 mM sodium acetate, pH 5.5, 250 mM NaCl. The protein was eluted at 2.0 ml/min, and aliquots (10ul) of each fraction were analyzed by RP-4 reverse phase chromatography and SDS-PAGE following the procedure of Example 2. Fractions containing correctly refolded IGF-1 C3 or IGF-1 C2 mutein monomer of 95% or more purity were pooled and concentrated to 250 ug/ml. This material was assayed for bioactivity and reacted with an 8.5 kDa polyethylene glycol as described below.

EXAMPLE 5

Pegylation of Muteins

The cysteine residue introduced into the 3rd position of the IGF-1 C3 mutein and the second position of the IGF-1 C2 mutein were covalently joined to an 8.5 kDa polyethylene glycol (8.5 kDa PEG) or an 20 kDa polyethylene glycol (20 kDa PEG) in a two step process:

1) The purified IGF-1 muteins were partially reduced with DTT in a 15 ml reaction mixture containing 2.3 mg (296 nmoles) IGF-1 mutein, 170 ug DTT (1110 nmoles) in 14 mM sodium acetate, 33 mM sodium phosphate, pH 7.0. The final concentration of protein was 10 ug/ml, and the molar ratio of DTT:protein was 3.75:1. The reaction mixture was incubated at room temperature for 3 hours and terminated by the addition of 1.0 ml of 1M sodium acetate, pH 5.5. The reaction mixture was dialyzed at 4°C overnight against 20 mM sodium acetate pH 5.5.

2) The partially reduced IGF-1 mutein was reacted with either the 8.5 kDa PEG or the 20 kDa PEG in a 20 ml reaction mixture containing 2.3 mg (296 nmoles) of protein, 9.985 mg (1174 nmoles) 8.5 kDa PEG in 15 mM sodium acetate, 26 mM sodium phosphate, pH 7.0. The final concentration of protein was 112 mg/l, and the molar ratio of 8.5 kDa PEG:protein was 4:1. The reaction mixture was incubated at room temperature for 3 hours, and terminated by placing at 4oC or freezing at -20°C. SDS-PAGE analysis of the reaction mixture following the procedure of Example 2 showed that approximately 50% of the partially reduced IGF-1 mutein was converted to a mono-pegylated species (pegylated C3 mutein or pegylated C2 mutein). The C3 and C2 20 kDa PEG and 8.5 kDa muteins migrated relative molecular weight of about 23 kDa and 60 kDa, respectively.

EXAMPLE 6

Purification of Pegylated Muteins

The pegylated C2 or C3 mutein reaction mixtures (containing approximately 100-200 mg protein) were dialyzed extensively at 4°C against 5 mM citric acid, pH 2.6. The pegylated mutein was separated from the unpegylated mutein using an S-Sepharose (Pharmacia/LKB, Piscataway, NJ) cation exchange column (2.5 X 25 cm) previously equilibrated with 5 mM citric acid buffer, pH 2.6. The bound protein was eluted with a 2000 ml linear gradient from 0 to 1 M NaCl. 25 ml fractions were collected. Pegylated C2 or C3 muteins eluted at 0.25-0.4M NaCl and the unpegylated protein eluted at 0.8-0.9 M NaCl.

Fractions containing the pegylated C2 or C3 muteins were pooled, concentrated and further purified by Sephacryl S-200 gel filtration chromatography. 15 ml of the concentrated fractions containing approximately 20 mg of total protein was loaded onto a Sephacryl S200 (Pharmacia/LKB, Piscataway, NJ) column (2.6 X 100 cm) previously equilibrated with 20 mM sodium acetate, pH 5.5 containing 250 mM NaCl. The protein was eluted at 2.0 ml per min. The bulk of the material eluted with an apparent MW of 200 kDa (on SDS-PAGE, 60 kDa).

EXAMPLE 7

Bioassay of Pegylated Muteins

Recombinant human metIGF-1 (rIGF-1) (Bachem California, Torrance, CA), the unpegylated C3 and C2 muteins and the pegylated C3 and C2 muteins were tested for their relative mitogenic activity and affinity for recombinant insulin-like growth factor binding protein 1 (IGF-BP1, which is described in PCT Application publication WO 89/09792, published on October 19, 1989.

A. Relative Mitogenic Activity

The relative mitogenic (growth stimulating) activities of the C3 and C2 muteins and pegylated C3 and C2 muteins were compared to that of wild type IGF-1 by measuring the relative amount of ^{3}H-thymidine incorporated into rat osteosarcoma cells when varying amounts of the proteins were present under serum free conditions. The rat osteosarcoma cells (the UMR106 cell line; obtained from American Type Culture Collection, Accession No. CRL-1661, Rockville, Maryland) were plated at 5-6 X 104 cells in 0.5 ml of Ham's F12 Media (Mediatech, Herndon, VA) containing 7% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and 2 mM L-glutamine per well in 48-well tissue culture plates (Costar, Cambridge, MA). After incubating for 72 hours at 37°C when the cells were confluent, the cells were washed twice with phosphate buffered saline (PBS) and pre-incubated in serum-free Ham's F12 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin and 2 mM L-glutamine for 24 hours. After the pre-incubation, 0.5 ml of F12 serum-free medium containing serial dilutions (1.0 - 1,000 ng/ml) of rIGF-1, C3 and C2 muteins, and pegylated C3 and C2 muteins were incubated separately for an additional 20-24 hours. Each well was then pulse labeled with 0.5 uCi of 3H-thymidine (NEN Research Products, DuPont Co., Boston, MA) for 4 hours, then washed with cold PBS three times and incorporated 3H-thymidine was precipitated with cold 7% trichloroacetic acid (J.T: Baker Inc., Phillipsburg, NJ). After rinsing with 95% ethanol, cells were solubilized with 0.3 M NaOH and aliquots removed for scintillation counting. 3H-thymidine was quantitated by liquid scintillation counting. All assays were performed in triplicate.

The C3 and C2 muteins and pegylated C3 and C2 muteins were found to stimulate the same maximal level of 3H-thymidine incorporation into DNA as recombinant IGF-1. The potencies of the C3 and C2 muteins and the pegylated C3 and C2 muteins were about 3 to 4 fold lower than recombinant IGF-1. The ED50 (dose required for half maximal activity) of recombinant IGF-1 was 5-10 ng/ml compared with 30-40 ng/ml for unpegylated C3 and C2 muteins, and the pegylated C3 and C2 muteins.

These experiments demonstrate that the mitogenic activity of IGF-1 has been substantially retained by the C3 and C2 muteins and the pegylated C3 and C2 muteins. All three molecules are capable of simulating cells to divide, as measured by ^{3}H-thymidine incorporation into DNA. All three molecules are capable of stimulating cells to divide to the same maximal extent.

B. Relative Affinity for IGF-BP1

The relative affinities of the C3 and C2 muteins and the pegylated C3 and C2 muteins for IGF binding protein-1 (IGF-BP1) were compared to that of the wild type IGF-1 by measuring the ability of IGF-BP1 to inhibit the mitogenic activities of the proteins on rat osteosarcoma cells. The rat UMR106 osteosarcoma cells were plated at 5-6 X 104 cells in 0.5 ml of Ham's F12 containing 7% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin and 2 mM L-glutamine per well in 48-well tissue culture plates. After incubating for 72 hours at 37°C when the cells became confluent, the cells were washed twice with PBS and pre-incubated in serum-free Ham's F12 medium containing 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine for 24 hours. After the pre-incubation, 0.5 ml of F12 serum-free medium containing either 50 ng/ml or 200ng/ml of rIGF-1, C3 or C2 mutein, or pegylated C3 or C2 mutein were incubated separately with varying amounts of IGF-BP1 (100 ng/ml - 1 X 104 ng/ml) for an additional 20-24 hours. Each well was then pulse labeled with 0.5 uCi of 3H-thymidine (NEN Research Products, Du Pont Co., Boston, MA) for 4 hours, then washed with cold PBS three times and incorporated 3H-thymidine was precipitated with cold 7% trichloroacetic acid (J.T. Baker Inc., Phillipsburg,

NJ). ^{3}H-thymidine was quantitated by liquid scintillation counting. All assays were performed in triplicate.

The results of this experiment indicated that the affinities of the unpegylated C3 mutein and the pegylated C3 mutein for IGFBP1 were greatly reduced. At a molar ratio of 20:1 (IGFBP1:rIGF-1), the mitogenic activity of rIGF-1 (50 ng/ml) was reduced 80%; however, the mitogenic activities of the same concentrations of the unpegylated C3 mutein and pegylated C3 mutein were reduced 35% and 0%, respectively. Similarly, when 200 ng/ml of the proteins were incubated with a 20 fold molar excess of IGF-BP1, 70% of the mitogenic activity of rIGF-1 was inhibited, whereas none of the mitogenic activity of the pegylated C3 mutein was inhibited. The affinities of of both the unpegylated C2 mutein and the pegylated C2 mutein were identical to that of wild type IGF-1.

These data indicate that the pegylated C3 mutein has greatly reduced affinity for IGFBP1 when compared to IGF-1. Thus the mitogenic activity of the pegylated C3 mutein will not be inhibited by IGF binding proteins under conditions where the mitogenic activity of IGF-1 will be inhibited. However, the affinity of pegylated C2 mutein for IGFBP1 is the same as the affinity of wild type IGF-1. Thus the mitogenic activity of pegylated C2 mutein will be inhibited by IGF binding proteins under the same conditions where the mitogenic activity of IGF-1 will be inhibited.

EXAMPLE 8

Animal Tests Protocols

A. Animals

Male Sprague Dawley rats with pituitary glands surgically removed (hypophysectomized or Hypox rats) and weighing 110-121 grams were obtained from Charles River Co. The rats were maintained in cages with lighting controlled over a 12 h-light/12 h-dark cycle.

The animals had continuous access to water and food. Five animals were housed per cage. The weights of the rats were monitored daily and only rats with weight gains of less than 2 grams per week during the 2-3 weeks after arrival

were considered to be succesfully hypophysectomized and used for the experiments.

B. Methods

In Experiment I, animals (10 Hypox rats per group) were injected every third day (ETD) subcutaneously (sc) with WT rIGF-I (160 mg, 320mg), unpegylated C2 (320mg), unpegylated C3 (320mg), pegylated C2 (C2-PEG, 320mg) or Pegylated C3 (C3-PEG,320mg) dissolved in 0.2 ml of binding buffer (0.1 M HEPES-0.05 M NaH_2PO_4). A separate group of 10 animals received 0.2ml vehicle. Injections were given between 0700 hours and 0800 hours and body weights were recorded daily between 1600 h and 1700 h. The weights of rats on the day after the last injection were taken as the final weight.

In Experiment II, animals (9 Hypox rats per group) were injected every third day sub-cutaneously with WT rIGF-I (320mg, single injection daily, SID; 320mg ETD; 640mg ETD), or C3-PEG (320mg ETD, 640mg ETD, 960mg ETD).dissolved in 0.2 ml of binding buffer (0.1 M HEPES-0.05 M NaH_2PO_4). A separate group of 9 animals received 0.2ml vehicle. Injections were given between 0700 h and 0800 h and body weight were recorded daily between 1600 h and 1700 h. The weights of rats on the day after the last injection were taken as the final weight.

In Experiment III, animals (10 Hypox rats) were injected every third day sub-cutaneously with C3-PEG (160mg ETD, 320mg ETD), dissolved in 0.2 ml vehicle (0.1 M HEPES-0.05 M NaH_2PO_4). A separate group of 10 animals received 0.2ml vehicle. Injections were given between 0700 h and 0800 h and body weight were recorded daily between 1600-1700 h. The weights of rats on the day after the last injection were taken as the final weight.

At the end of Experiments I & II, rats were asphyxiated with CO_2 and weighed. In Experiment III, the tibia were removed and the epiphyseal width measured.

C. Results

Experiment I: Rats treated with sc injections with either 160mg or 320mg of WT IGF-I ETD showed no significant weight gain compared with animals

injected with vehicle (Table 2). Similarly, animals injected ETD with 320 mg un-PEGylated C2 IGF-I mutein did not show significant weight gain. However, un-PEGylated C3 IGF-I mutein (320 mg ETD) stimulated 1.75 ± 0.90 g of growth. Although this weight gain was not statistically significant, a clear trend towards increased growth was observed. Animals injected ETD with 320 mg C2-PEG and C3-PEG gained 4.42 ± 0.74 g and 5.45 ± 0.98 g, respectively. The PEGylated proteins clearly showed efficacy; however, the identical dose of WT IGF-I showed no efficacy.

TABLE 2

THE EFFECT OF IGF-I MUTEINS (UNPEGYLATED & PEGYLATED) ON THE GROWTH OF HYPOPHESECTOMIZED RATS

MOLECULE	DOSE ug/day	FREQUENCY	MEAN WT GAIN (g)	P VALUE vs
Vehicle		ETD	-1.28 ± 0.95	
WT IGF-I	160	ETD	0.23 ± 0.87	
WT IGF-I	320	ETD	0.59 ± 0.79	
C2	320	ETD	-0.52 ± 0.67	
C2-PEG	320	ETD	4.42 ± 0.74	WT 320 0.01 C2 320 0.01 C3 320 <0.05
C3	320	ETD	1.75 ± 0.90	
C3-PEG	320	ETD		WT 320 <0.01 C2 320 <0.05 C3 320 < .05

Experiment I demonstrates that the mean weight gain of animals treated with un-PEGylated C3 ETD (1.75g ± 0.90g) was greater than the mean weight gain of animals treated with un-PEGylated C2 ETD (-0.52g ± 0.67g). Similar to un-PEGylated C2, animals treated with WT IGF-I ETD failed to gain weight. Since, *in vitro*, un-PEGylated C2 and C3 have identical bioactivities, the apparent greater *in vivo* bioactivity of un-PEGylated C3 in this experiment reflects the *in vitro* observation that un-PEGylated C3 has greatly reduced affinity for IGF-BP1. It has been reported that IGF-I muteins with reduced affinity for IGF binding proteins exhibit greater *in vivo* bioactivity than WT-IGF-I. Un-PEGylated C2, however, has unaltered affinity for IGF-BP1, and similar bioactivity to WT IGF-I. Both C2-PEG and C3-PEG have significantly greater potency than WT IGF-I and both un-PEGylated muteins. These results demonstrate that the PEGylated muteins exhibit enhanced pharmacodynamics over WT IGF-I.

Experiment II: Rats treated with sc injections of WT IGF-I 320mg SID, 320mg ETD and 640mg ETD gained 4.02g ± 0.46g , 0.81g ± 0.81g and 1.41g ± 0.52g, respectively (Table 3). However, animals given 160mg, 320mg, 640mg 960mg of C3-PEG ETD gained 5.22g + 0.46g, 5.50g ± 0.52g, 8.69g ± 0.67g, and 10.43g ± 0.77g, respectively (Table 3). All doses of C3-PEG ETD stimulated significantly more weight gain than both WT IGF-I doses given ETD. Animals injected with either 640mg or 960mg of C3-PEG ETD gained significantly more weight than those given 320mg WT IGF-I SID. C3-PEG doses of 160mg and 320mg ETD clearly stimulated greater weight gain than 320mg of WT IGF-I SID; however, these differences did not reach statistical significance.

TABLE 3

THE EFFECT OF C3-PEGylated IGF-I ON THE GROWTH OF HYPOPHESECTOMIZED RATS

MOLECULE	DOSE ug/day	FREQUENCY	MEAN WT GAIN (g)	P VALUE vs
Vehicle		ETD	-0.22 ± 0.38	
WT IGF-I	320	SID	4.02 ± 0.46	WT 320 ETD 0.01 WT 640 ETD 0.01
WT IGF-I	320	ETD	0.81 ± 0.81	
WT IGF-I	640	ETD	1.41 ± 0.52	
C3-PEG	160	ETD	5.22 ± 0.46	WT 320 ETD 0.01 WT 640 ETD 0.01
C3-PEG	320	ETD	5.50 ± 0.52	WT 320 ETD 0.01 WT 640 ETD 0.01
C3 PEG	640	ETD	8.69 ± 0.67	WT 320 SID <0.01 C3PEG 160 ETD <0.01 C3PEG 320 ETD <0.01
C3-PEG	960	ETD	10.43 ± 0.77	WT 320 SID <0.01 C3PEG 160 ETD <0.01 C3PEG 320 ETD <0.01

Experiment II demonstrates that C3-PEG administered sc ETD exhibits greater potency than WT IGF-I administered sc ETD. All doses of C3-PEG stimulated greater mean weight gain than animals given 320mg WT IGF-I SID.

The enhanced pharmacodynamics of C3-PEG make it more potent than WT IGF-I in the animal model described.

Experiment III: Rats treated with sc injections of C3-PEG 160 mg and 320 mg gained 8.3g ± 0.7g and 9.0g ± 0.6g, respectively (Table 4). Vehicle gained 4.2g + 0.3g. The weight gained induced by C3-PEG was statistically greater than animals given vehicle. Similarly, the tibial epiphyseal widths of rats receiving C3-PEG were statistically greater than rats receiving vehicle (Table 4).

TABLE 4

THE EFFECT OF C3-PEGylated IGF-I ON THE GROWTH OF HYPOPHESECTOMIZED RATS (WEIGHT GAIN)

MOLECULE	DOSE ug/day	FREQUENCY	MEAN WT GAIN (g)	P VALUE vs
Vehicle		ETD	4.2 ± 0.3	
C3-PEG	160	ETD	8.3 ± 0.46	Vehicle <0.01
C3-PEG	320	ETD	9.0 ± 0.g	Vehicle <0.01

THE EFFECT OF C3-PEGylated IGF-I ON THE GROWTH OF HYPOPHESECTOMIZED RATS (TIBIA EPIPHYSEAL WIDTH)

MOLECULE	DOSE ug/day	FREQUENCY	MEAN TIBIAL WIDTH (mm)	P VALUE vs
Vehicle		ETD	0.136 ± 0.008	
C3-PEG	160	ETD	0.180 ± 0.01	Vehicle <0.01
C3-PEG	320	ETD	0.167 ± 0.06	Vehicle <0.01

Experiment III demonstrates that C3-PEG stimulates not only weight gain, but also bone growth in HYPOX rats. This indicates that C3-PEG may be a useful pharmaceutical for the induction of bone formation.

Although this invention has been described with respect to specific embodiments, it is not intended to be limited thereto. Various modifications which will be apparent to those skilled in the art are deemed to fall within the spirit and scope of the present invention.

Claims:

1. A polyethylene glycol (PEG) conjugate comprising PEG and a mutein of IGF, said PEG attached to said mutein at a free cysteine in the N-terminal region of the mutein.

2. The conjugate of claim 1, wherein said PEG is attached to the free cysteine through an activating group selected from the group consisting of maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane, and 5-pyridyl.

3. The conjugate of claim 1, wherein the IGF is IGF-1.

4. The conjugate of claim 3, wherein the free cysteine occurs before the first amino acid from the N-terminus of IGF-1.

5. The conjugate of claim 4, wherein the free cysteine is adjacent to the first amino acid from the N-terminus of IGF-1.

6. The conjugate of claim 3, wherein the free cysteine occurs between any two adjacent amino acids of approximately the first twenty amino acids from the N-terminus of IGF-1.

7. The conjugate of claim 3, wherein the free cysteine is a substitute for any of approximately the first twenty amino acids from the N-terminus of IGF-1.

8. The conjugate of claim 7, wherein the free cysteine is a substitute for the first amino acid from the N-terminus of IGF-1.

9. The conjugate of claim 7, wherein the free cysteine is a substitute for the second amino acid from the N-terminus of IGF-1.

10. The conjugate of claim 7, wherein the free cysteine is a substitute for the third amino acid from the N-terminus of IGF-1.

11. The conjugate of claim 1, wherein the PEG has a molecular weight selected from the group consisting of 5 kDa, 8.5 kDa, 10 kDa, and 20 kDa.

12. The conjugate of claim 11, wherein the PEG has a molecular weight of 8.5 kDa.

13. The conjugate of claim 1, further comprising a second polypeptide attached to said PEG.

14. The conjugate of claim 13, wherein the second polypeptide is a mutein of IGF.

15. A mutein of IGF having a free cysteine in the N-terminal region of the mutein.

16. The mutein of claim 15, wherein said mutein is a recombinant product.

17. The mutein of claim 16, wherein said mutein is expressed by E. coli.

18. A method of making the conjugate of claim 1, comprising attaching PEG to a free cysteine of an IGF mutein, said mutein having a free cysteine in the N-terminal region.

19. The method of claim 18, wherein said PEG is attached to the free cysteine through an activating group selected from the group consisting of maleimide, sulfhydryl, thiol, triflate, tresylate, aziridine, exirane, and 5-pyridyl.

20. The method of claim 19, wherein the activating group is maleimide.

21. The method of claim 18, wherein the PEG is attached to an IGF mutein and another polypeptide.

22. The method of claim 21, wherein the other polypeptide is an IGF mutein.

23. A pharmaceutical composition comprising the conjugate of claim 1 in a pharmaceutically acceptable carrier.

24. A method for treating an IGF associated condition comprising administering a PEG conjugate of IGF of claim 1 to a patient.

25. The method of claim 24, wherein said PEG conjugate is administered to the patient in pharmaceutically acceptable carrier.